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Deterioration of Phospholipids of Skipjack Muscle during Ice Storage: Mainly Concerning to Enzymatic Hydrolysis of Phosphatidylcholine

Toshiaki OHSHIMA,* Shun WADA,* and Chiaki KOIZUMI*

Changes in phospholipids of skipjack muscle during ice storage were studied using GLC, HPLC, and TLC. During ice storage, the contents of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SPM), and phosphatidylserine (PS) decreased, while those of lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and free fatty acid (FFA) increased. These results indicate that enzymatic hydrolysis of phospholipids occurred, since the content of triglyceride did not change.

LPC and LPE were found in the muscle of skipjack analyzed the day after the catch, at though less in quantity, but increased during ice storage for 2 days, and then decreased. The maximum amount of LPC was 99.5 mg/100 g. In the fatty acid composition of LPC, percentages of $C_{16:0}$, $C_{18:1}$, $C_{18:0}$, and $C_{18:1}$ were relatively high, while that of $C_{22:6}$ extremely low, though percentage of $C_{22:6}$ was highest in the fatty acid composition of PC. In addition, percentage of the PC having two molecules of $C_{22:6}$ as constituent fatty acids increased gradually during ice storage. These results suggest that the PC having $C_{22:6}$ on α-position of glycerol was scarcely attacked by the action of phospholipase A_2 in the skipjack muscle.

It has long been known that phospholipids in fish decrease as they undergo enzymatic hydrolysis during low-temperature storage and that accompanying this process free fatty acid increases.[1-10] Moreover, it is said that many enzymes play a part in the hydrolysis of phospholipids and that there is no accumulation of the intermediate product, lysophospholipids.[6,7,11-13]

However, as shown in a previous report,[14] there are large amounts of lysophospholipids in the muscle tissue of skipjacks sold fresh at the market-place, in particular, there is an accumulation of lysophosphatidycholine (LPC). Generally, it is held that enzymatic

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hydrolysis of phospholipids occurs most rapidly at -4 degrees C.,[15,16] and the fact that a large amount of LPC existed in fresh skipjack may be thought to indicate that in skipjack which is is refrigerated or even in ice storage, phosphatidycholide (PC) undergoes rapid hydrolysis.

The present research, intended as one link in the research clarifying the mechanism of enzymatic hydrolysis of phospholipids in skipjack muscle, utilized fresh skipjack with a clear after-catch history and investigated the conditions of accumulation of lysophospholipids during ice storage, LPC and PC fatty acid composition changes, and changes in the composition of PC fatty acid.

Experimental Method

Fish used. Five fresh skipjack (Euthynnus pelamis), average weight 1.7 kg, landed at Numatsu Harbor, Shizuoka Prefecture, in early June 1982, were packed in ice an brought to the laboratory on the second day after being caught. One of these was immediately examined to provide Day-0 data. The remaining fish were individually sealed in polyethylene bags packed in ice in a refrigerator room at 4 degrees C. and one fish examined every second day.

Extraction of Lipids. After removal of head, viscera bones, skin and dark muscle, , the ordinary muscle was minced, and, in the same manner as in the previous report,[14] the total fat (TL) was extracted by the Bligh and Dyer method.[17] Attempts were made during preparation to mix in as little

subcutaneous fat as possible.

Fat fractionation PL's HPLC, Densitometry, Estimation of PC Fatty Acid Constituents. All carried out as in previous report.[14)]

Results and Conclusions

Changes in Fat Amounts. The change in fat content of ordinary skipjack muscle during ice storage is shown in Table 1. The lipid content is relatively low. The amount of lipids found on Day-0 was 759/100g and included PL amounts of 559mg/100g, about 79% of TL. This is rather lower than the TL content 1214
of 1090mg/100g found in fresh, commercial ordinary skipjack muscle in the previous report,[14)] but it was thought that the amount of subcutaneous fat removed influenced this result.

With storage, TL and PL decreased while NL showed a tendency to increase. In this research, because FFA was fractionated as NL, the increase in NL which accompanied the decrease in PL indicates that the production of FFA was dependent on the hydrolysis of PL. NL and PL composition ratios until Day-4 and those after Day-6 differed markedly. Judging from the VBN measurement results, it may be thought that after Day-6 of storage (VBN 17.1 mg/100g) microorganisms influenced the result.

TL, NL and Fatty Acid Composition Changes. Changes in the fatty acid composition of TL, NL and PL are shown in Tables 2, 3, and 4, respectively. In the TL fatty acid 1215
composition, $C_{22:6}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ composition ratios were high, but, except for $C_{18:0}$, there was almost no change. It can be thought that this fact shows that almost no

fat oxidation occurred during storage. In fact, there was almost no increase in TBA value[18] during storage. $C_{18:0}$, and, although low in composition ratio, $C_{22:4}$, increased slightly during storage. The fatty acid composition of NL was similar in variety to TL, but, except for Day-6, $C_{16:0}$ had a higher composition ratio than $C_{22:6}$. During storage the composition ratio of $C_{16:0}$ showed a tendency to decrease while $C_{22:6}$ displayed an increasing tendency. The fact that the fatty acid composition of NL changed during storage is due to the increase in FFA produced through the hydrolysis of various phospholipids besides triglyceride (TG) and sterol ester.

The main constituents of fatty acid composition of PL are $C_{22:6}$, $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$. The fatty acids which experienced composition ratio decreases during storage are $C_{16:0}$ and $C_{20:5}$. On the other hand, odd-numbered fatty acids such as $C_{15:0}$, $C_{17:0}$, etc. had a tendency to increase slightly.

The change in fatty acid composition of NL and PL until Day-4 and that after Day-6 differed markedly. It can be conjectured that after the sixth day microorganisms influenced the fatty acid composition.

Changes in HPLC Patterns of PL. Changes in HPLC patterns during storage are shown in Fig. 1. As stated in the previous report,[14] under the HPLC conditions of the present research, five varieties of phospholipids, phophatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), PC, sphingomyelin (SPM), and LPC are eluted, in this order. 1216

The skipjack examined in this experiment was very fresh fish landed two days after being caught, immediately examined, and used as Day-0 data. However, clear LPE and LPC peaks were already recognizable in the Day-0 HPLC chromatogram. These lysophospholipid peaks increase in size with storage time, but, after the sixth day of storage, the PC and LPC peaks became smaller and their shapes altered radically. This may be thought due to rapid hydrolysis of PC and LPC. As there is a strong possibility that microorganisms became involved in the phospholipid changes from Day-6 forward, only samples until Day-4 of storage are analyzed below.

Changes of Constituents of NL and PL. From the results obtained from densitometry and TLC, and from the NL and PL contents shown in Table 1, the amount of each lipid constituting NL and PL per one hundred grams of fish muscle were calculated.

The respective changes in these during storage are shown in Figs. 2 & 3. Among the changes in constituents of NL during storage, FFA amounts increased, but, on the other hand, quantities of TG did not decrease. Thus, the increase in FFA during storage depended on hydrolysis of PL. That is, as shown in Fig. 3, PC (since, under the conditions of TLC in this experiment, LPE is not completely separated out, LPE is included), PE, SPM, and PS all decreased and LPC alone displayed a tendency to increase. It may be conjectured that these phospholipid changes are all mainly due to hydrolysis. The LPC amount reached its

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greatest value (99.5mg/100g) on the second day of storage, but this accumulation when compared to the amount found in fresh, commercial skipjack (144mg/100g) in the previous report is quite low.

As shown above, it is generally said that phospholipid hydrolysis progresses most rapidly at -4 degrees C., but in the case of skipjack even during ice storage (fish body-temperatures are thought to have been slightly above 0 degrees C.) it progressed rapidly and lysophospholipid accumulation was evident.

Changes in PC, LPC and FFA Fatty Acid Composition. Changes in fatty acid composition of PC, LPC and FFA are shown in Tables 5, 6 & 7, respectively. Furthermore, using these fatty acid compositions and the amounts of each class of lipids shown in Fig. 3, the amounts of the principal fatty acids per one hundred grams of fish muscle were calculated and those changes are shown in Figs. 4, 5 & 6.

As shown in Table 5, the principal constituent fatty acids of PC are $C_{22:6}$, $C_{16:0}$, $C_{18:1}$ and $C_{20:5}$. During storage $C_{16:0}$ and the odd-numbered carbon fatty acid $C_{15:0}$ composition ratios gradually decreased and $C_{22:6}$ and $C_{22:5}$ slightly increased. However, when viewed in terms of weight changes of constituent fatty acids, as shown in Fig. 4, $C_{22:6}$ is clearly decreasing.

$C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ were the main constituent fatty acids in the fatty acid composition of LPC. As in

the LPC of fresh, commercial skipjack recorded in the previous report, the composition ratios of $C_{20:5}$ and $C_{22:6}$ were markedly low. During storage the composition ratio of saturated fatty acids such as $C_{16:0}$ and $C_{18:0}$ and highly unsaturated* fatty acids $C_{22:4}$, $C_{22:5}$, $C_{22:6}$, gradually decreased while the composition ratios of $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:4}$ and the odd-numbered carbon fatty acid $C_{15:0}$ showed a tendency to increase. As shown in Fig. 5, when viewed in terms of weight change of constituent fatty acids, after temporarily increasing, both $C_{16:0}$ and $C_{18:1}$ showed a tendency to decrease.

In the fatty acid composition of FFA the composition ratio of $C_{16:0}$ was markedly high and composition ratios for $C_{22:6}$, $C_{18:0}$, etc. were also high. Since, as shown in Fig. 2, the TG content of NL did not decrease during storage, it can be thought that FFA is principally derived from various phospholipids and there are almost no TG derivatives. During storage the composition ratios of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, etc. showed a tendency to decrease while those of $C_{16:1}$, $C_{20:5}$ and $C_{22:6}$ increased. However, as shown in Fig. 6, when viewed in terms of weight changes of principal fatty acids, $C_{16:0}$ showed a slight tendency to increase.

Fatty Acid Constituents of PC. As in previous reports,[14,19] PC fatty acid constituents were estimated from

the fatty acid composition of hydrogenated PC (Table 8) and from the PC composition (Table 9); the changes during storage are shown in Table 10.

On Day-0 of storage (C_{16},C_{22}) had the highest composition ratio followed in order by (C_{18},C_{22}), (C_{16},C_{18}), (C_{16},C_{20}), (C_{22},C_{22}) and (C_{20},C_{22}). Estimating from the PC fatty acid composition of Table 5, the larger part of (C_{16},C_{22}), (C_{18},C_{22}), (C_{16},C_{18}), (C_{16},C_{20}), (C_{22},C_{22}), and (C_{20},C_{22}) may be thought to form from ($C_{16:0}$,$C_{22:6}$) ($C_{18:1}$,$C_{22:6}$), ($C_{16:0}$,$C_{18:1}$), ($C_{16:0}$,$C_{20:5}$) or ($C_{16:0}$,$C_{20:4}$), ($C_{22:6}$,$C_{22:6}$) and ($C_{20:5}$,$C_{22:6}$) or ($C_{20:4}$,$C_{22:6}$), respectively. 1218

During storage the composition ratio of (C_{16},C_{18}) decreased and the composition ratios of (C_{22},C_{22}) and (C_{18},C_{20}) showed a tendency to increase. As stated earlier, it is estimated from the PC fatty acid composition that most of (C_{22},C_{22}) formed from ($C_{22:6}$,$C_{22:6}$). This may be thought to indicate that the increase during storage of the composition ratio of ($C_{22:6}$,$C_{22:6}$) may be because PC having this composition did not undergo hydrolysis by Phospholiphase A_2, or, that if hydrolysis did occur, it was very slight. This is inferred from the fact that, as shown in Table 5, during storage the composition ratio of $C_{22:6}$in PC gradually increased and the fatty acid composition of LPC shown in Table 6 contains almost no $C_{22:6}$.

However, because the influence of microorganisms appeared earlier than foreseen in this research, it was not possible to continue the experiment until the enzymatic hydrolysis of the phospholipids had progressed sufficiently.

Next time, in order to avoid the effects of microorganisms, we hope to perform the experiment at low temperatures and clarify the mechanism of phospholipid hydrolysis in fish muscle at the molecular level.

We thank Mr. Yamazaki Shogo who cooperated in the course of this experiment.

Tables and Figures

Table 1. Changes in the contents of TL, PL, and NL of Skipjack muscle during ice storage (mg/100 g)

Storage, day	TL*	PL*	NL*
0	759	599	156
2	775	512	263
4	702	506	196
6	626	180	446
8	689	313	376

* TL, Total lipid; PL, Polar lipid; NL, Non-polar lipid.

Table 2. Changes in fatty acid composition of the TL during ice storage (wt %)

Fatty acid	Storage, day				
	0	2	4	6	8
14:0	0.5	0.8	0.6	0.6	0.3
:1	tr	tr	tr	tr	tr
15:0	0.4	1.9	1.5	1.9	0.5
16:0	22.0	21.5	22.5	22.0	23.7
:1	1.4	1.9	2.0	1.8	1.3
:2	0.8	0.8	0.7	0.4	1.7
17:0	1.0	1.8	1.2	0.9	1.6
18:0	10.4	10.2	11.1	12.1	13.1
:1	8.9	9.3	10.3	11.0	10.3
:2	0.7	1.0	1.4	1.0	0.9
:3	0.8	0.8	0.6	0.8	0.8
19:0	—	—	—	tr	tr
20:0	0.3	0.5	0.5	0.3	0.3
:2	0.3	0.3	0.2	0.3	0.3
:4	4.2	3.2	3.9	3.9	3.7
:5	5.5	4.9	4.6	4.4	5.2
21:0	0.4	0.2	0.5	0.3	0.2
22:4	0.1	1.0	1.2	1.5	1.6
:5	7.2	3.0	3.3	2.7	2.3
:6	34.0	36.6	33.6	33.9	31.9
23:0	1.2	0.3	0.2	0.3	0.3

Table 3. Changes in fatty acid composition of the NL during ice storage (wt %)

Fatty acid	Storage, day				
	0	2	4	6	8
14:0	1.7	1.3	1.3	0.6	0.6
:1	tr	tr	tr	tr	tr
15:0	0.7	0.6	0.8	0.4	0.5
16:0	38.9	34.9	37.2	25.0	32.4
:1	3.3	2.7	3.1	1.9	1.9
:2	0.8	0.7	0.5	0.3	0.6
17:0	1.8	1.7	2.0	1.5	1.5
18:0	12.1	11.7	11.4	13.0	13.0
:1	8.7	8.9	9.8	9.4	7.1
:2	1.3	1.1	1.6	1.3	1.0
:3	1.0	1.1	1.1	0.8	0.7
19:0	—	—	—	tr	tr
20:0	0.3	0.5	0.8	0.4	0.4
:2	0.3	0.2	0.2	0.2	0.2
:4	2.3	2.2	2.2	3.0	3.0
:5	4.5	5.0	6.0	5.4	5.6
21:0	1.2	0.5	0.4	0.2	0.2
22:4	0.1	0.7	0.5	0.4	0.4
:5	3.4	1.6	1.9	2.3	2.0
:6	16.6	24.2	18.6	33.7	28.5
23:0	0.9	0.3	0.7	0.2	0.2

Table 4. Changes in fatty acid composition of the PL during ice storage

(wt %)

Fatty acid	Storage, day				
	0	2	4	6	8
14:0	0.6	0.4	0.3	1.4	0.7
:1	tr	tr	tr	tr	tr
15:0	1.9	1.9	2.4	5.7	6.9
16:0	18.8	15.0	17.7	19.8	10.6
:1	1.4	1.3	1.3	1.8	1.4
:2	0.5	0.4	0.6	0.4	1.1
17:0	0.8	0.7	1.9	2.9	2.8
18:0	9.0	10.2	9.6	18.2	8.4
:1	10.4	12.3	11.1	18.1	13.3
:2	0.8	0.8	1.0	0.5	0.8
:3	0.8	0.6	0.6	—	0.6
19:0	—	—	—	tr	tr
20:0	0.5	0.2	0.2	0.9	0.3
:2	0.7	0.4	0.2	—	0.3
:4	5.4	4.4	4.0	3.1	5.3
:5	6.5	6.7	5.3	2.0	4.7
21:0	0.5	tr	tr	—	0.3
22:4	0.3	0.8	0.3	1.3	1.1
:5	3.6	3.3	4.3	2.1	2.6
:6	35.0	39.7	39.1	20.9	39.1
23:0	2.6	0.8	tr	0.7	0.3

Table 5. Changes in fatty acid composition of the PC during ice storage

(wt %)

Fatty acid	Storage, day		
	0	2	4
14:0	0.3	0.4	0.2
:1	tr	tr	tr
15:0	2.7	2.6	2.0
16:0	25.3	21.1	22.6
:1	1.4	1.6	1.4
:2	0.6	0.5	0.4
17:0	1.4	1.4	0.7
18:0	3.0	4.3	3.1
:1	8.5	9.0	9.0
:2	0.6	0.7	0.7
:3	0.4	0.4	0.4
20:0	0.3	0.3	0.3
:2	0.1	0.2	0.2
:4	3.7	3.7	4.0
:5	7.2	6.8	7.5
22:0	0.1	0.1	0.1
:4	0.2	0.5	0.3
:5	2.8	3.1	3.9
:6	41.5	43.4	42.8
23:0	—	—	0.5

Table 6. Changes in fatty acid composition of the LPC during ice storage

(wt %)

Fatty acid	Storage, day		
	0	2	4
14:0	4.7	7.2	5.4
:1	3.1	2.8	—
15:0	3.5	2.8	6.4
16:0	35.3	27.9	28.7
:1	7.6	7.9	13.2
:2	tr	6.1	1.9
17:0	tr	tr	1.1
18:0	10.9	9.6	8.0
:1	12.9	18.5	18.2
:2	0.8	2.3	3.5
:3	tr	tr	4.1
20:0	2.4	2.9	2.9
:2	tr	0.3	0.3
:4	1.9	3.3	4.0
:5	tr	tr	tr
22:0	4.8	4.9	—
:4	5.6	0.6	0.3
:5	3.6	2.4	1.2
22:6	3.0	0.4	0.8

Table 7. Changes in fatty acid composition of the FFA during ice storage

(wt %)

Fatty acid	Storage, day		
	0	2	4
14:0	0.4	0.4	0.5
:1	0.1	tr	tr
15:0	0.6	0.6	0.7
16:0	48.9	44.8	44.7
:1	0.5	1.9	2.1
:2	0.4	1.0	0.7
17:0	1.2	2.3	1.7
18:0	16.6	18.1	12.7
:1	6.2	7.2	5.9
:2	1.0	0.8	1.0
:3	0.6	0.6	0.6
20:0	0.3	0.3	0.3
:2	tr	0.2	0.1
:4	1.2	1.3	1.7
:5	2.9	2.6	4.0
21:0	0.2	—	0.1
22:0	—	tr	—
:4	—	0.1	0.1
:5	1.1	1.1	1.2
:6	17.7	16.3	21.7
23:0	—	0.2	0.2

Table 8. Changes in fatty acid composition of the PC after hydrogenation

(mol %)

Fatty acid	Storage, day		
	0	2	4
14:0	0.4	0.8	0.5
15:0	2.8	2.6	0.4
16:0	31.2	26.2	29.8
17:0	1.3	1.9	0.8
18:0	12.5	14.3	13.6
19:0	0.2	0.7	—
20:0	9.7	10.7	10.8
21:0	0.1	0.4	—
22:0	41.7	42.4	44.1

Table 9. Changes in PC composition based on the total carbon in fatty acyl chains after hydrogenation

(mol %)

TC*	Storage, day		
	0	2	4
32	2.7	1.7	1.0
33	1.7	1.6	—
34	13.7	6.7	8.6
35	1.3	2.3	—
36	11.7	10.7	12.7
37	2.5	5.8	—
38	45.4	40.6	46.5
40	12.3	15.4	12.3
42	4.1	5.8	5.7
44	6.0	9.5	13.1

* TC, Total carbon in fatty acyl chains of the PC

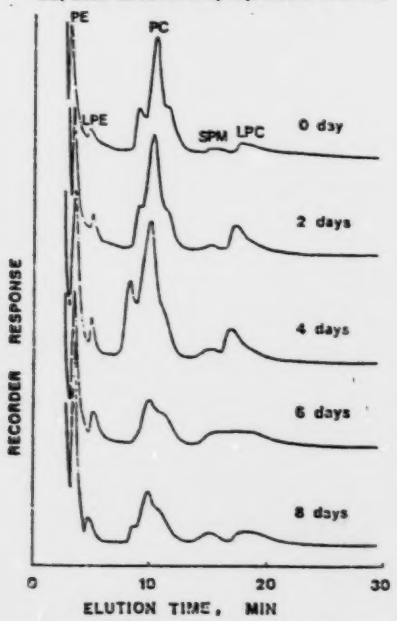

Fig. 1. Changes in the elution patterns of phospholipids of skipjack muscle in HPLC during ice storage.

Table 10. Possible fatty acid combinations of the PC and changes in their percentages during ice storage

(mol %)

TC	Combination of Fatty acid	Storage, day		
		0	2	4
32	C_{14}, C_{18}	0.5	0.2	0.1
	C_{15}, C_{17}	1.7	0.2	0.9
	C_{16}, C_{16}	0.5	1.4	0.1
33	C_{14}, C_{19}	0.4	0.1	—
	C_{15}, C_{18}	1.0	0.1	—
	C_{16}, C_{17}	0.4	1.3	—
34	C_{14}, C_{20}	0.8	0.2	0.1
	C_{15}, C_{19}	0.9	0.1	—
	C_{16}, C_{18}	10.6	5.4	8.1
	C_{17}, C_{17}	1.4	1.0	0.5
35	C_{14}, C_{21}	0.1	0.1	—
	C_{15}, C_{20}	1.1	0.1	—
	C_{16}, C_{19}	0.1	1.2	—
	C_{17}, C_{18}	0.1	0.8	—
36	C_{14}, C_{22}	2.2	2.3	1.4
	C_{15}, C_{21}	0.1	0.1	—
	C_{18}, C_{20}	9.3	8.1	10.4
	C_{17}, C_{19}	tr	tr	—
	C_{18}, C_{18}	tr	0.2	0.8
37	C_{15}, C_{22}	2.9	5.7	—
	C_{16}, C_{21}	—	tr	—
	C_{17}, C_{20}	tr	0.1	—
38	C_{18}, C_{22}	44.3	34.4	41.4
	C_{19}, C_{20}	1.6	6.3	5.1
40	C_{18}, C_{22}	12.7	15.6	12.4
	C_{20}, C_{20}	0.1	—	—
42	C_{20}, C_{22}	4.9	6.0	5.9
44	C_{22}, C_{22}	7.2	10.0	13.4

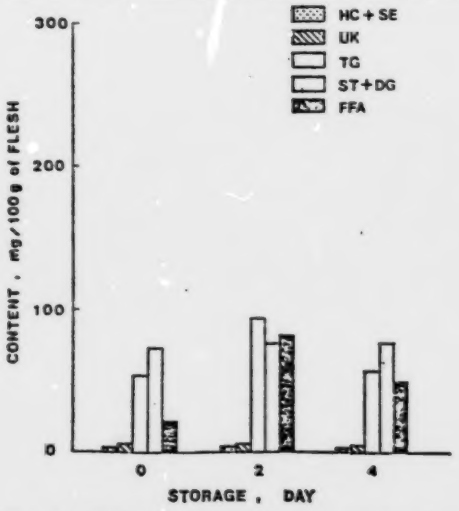

Fig. 2. Changes in the contents of lipid classes in the NL during ice storage; HC, hydrocarbon; SE, sterol ester; UK, unknown compound; TG, triglyceride; ST, free sterol; DG, diglyceride; FFA, free fatty acid.

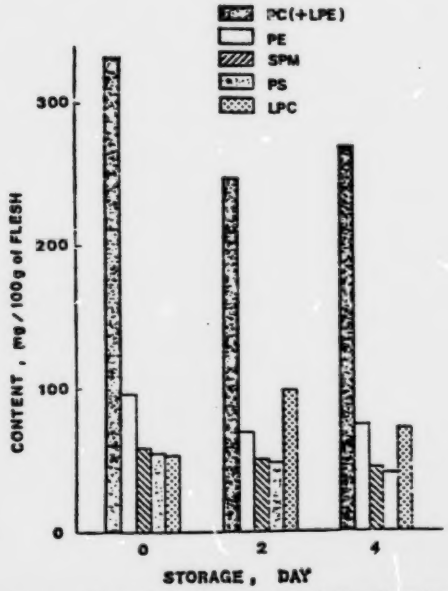

Fig. 3. Changes in the contents of phospholipids in the PL during ice storage. PC, phosphatidylcholine; LPE, lysophosphatidylcholine; PE, phosphatidylethanolamine; SPM, sphingomyelin; PS, phosphatidylserine; LPC, lysophosphatidylcholine.

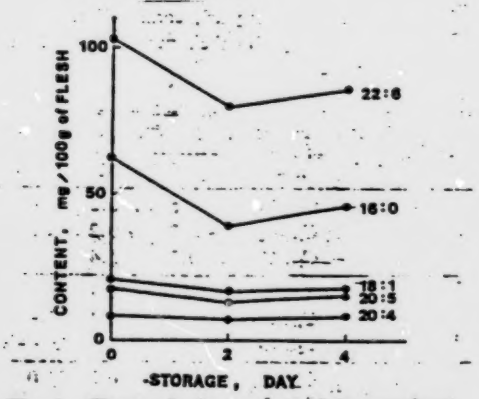

Fig. 4. Changes in the contents of the prominent fatty acids of the PC during ice storage.

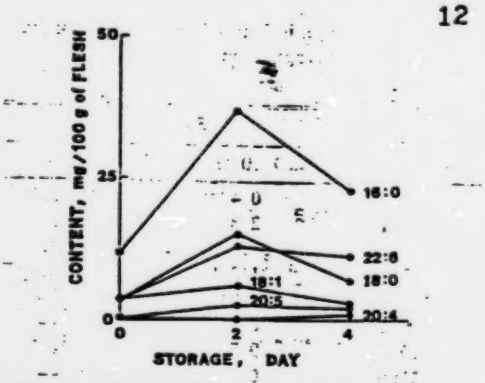

Fig. 6. Changes in the contents of the prominent fatty acids of the FFA during ice storage.

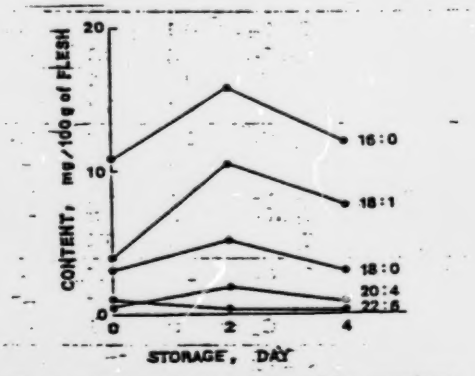

Fig. 5. Changes in the contents of the prominent fatty acids of the LPC during ice storage.

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